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Phylogeny of Aspleniaceae Inferred from *rbcL* Nucleotide Sequences

NORIAKI MURAKAMI[1]
Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake-cho, Kyoto 606-8502, Japan
SATORU NOGAMI
Department of Plant Sciences, School of Science, University of Tokyo, Hongo, Tokyo 113-0033, Japan
MIKIO WATANABE
Department of Biology, Aichi Kyoiku University, Kariya 448-0001, Japan
KUNIO IWATSUKI
Faculty of Science, Rikkyo University, Tokyo 171-0021, Japan

ABSTRACT.—We determined *rbcL* sequences of 25 species and 2 varieties of Aspleniaceae with various leaf and rhizome morphologies, and conducted a phylogenetic analyses with the following conclusions: 1) leaf shape is not congruent with *rbcL* phylogeny in Aspleniaceae; 2) rhizome morphology (erect-ascending or creeping) reflects *rbcL* phylogeny; 3) naturally occurring hybrids are generated only between closely related species and thus reflect the *rbcL* phylogeny. The third conclusion was especially well-supported by our allozyme analyses of hypothesized hybrids between distantly related species of Aspleniaceae. A popular cultivated fern hybrid in Japan, *Asplenium ×kenzoi,* is believed to be a hybrid between *A. prolongatum* and *A. wrightii,* which are distantly related in our molecular tree. However, our allozyme analysis of *A. ×kenzoi* showed that it is a hybrid between *A. antiquum* and *A. prolongatum,* whose close relationship was first suggested by our *rbcL* tree. Thus, *A. ×kenzoi* appears to to be a hybrid between two closely related species with very different morphologies.

Aspleniaceae are a well-defined family of leptosporangiate ferns, characterized by an elongate sorus type along the leaf veins with an elongate indusium, X-shaped leaf traces in the upper parts of fronds, clathrate rhizome scales, and a basic chromosome number of x = 36. However, intrafamiliar relationships of the Aspleniaceae are poorly known and no widely accepted system of classification for the family has been established (Iwatsuki, 1975; Tryon and Tryon, 1982). More than 700 species currently belong to one genus, *Asplenium* L., although various authors have segregated some species of *Asplenium* into different genera such as *Phyllitis* Newm., *Camptosorus* Rupr., *Neottopteris* J.Sm., *Boniniella* Hayata, and *Hymenasplenium* Hayata. The status of these genera is too obscure to be widely accepted because the relationship to other members of the Aspleniaceae is not clear.

In this study, we determined the *rbcL* sequences of 25 species of Aspleniaceae with various leaf and rhizome morphologies and conducted a molecular phylogenetic analysis in order to elucidate intrafamiliar relationships. A mo-

[1] Author for correspondence.

lecular phylogeny has already been constructed for a portion of the family, the *Hymenasplenium* group, using both restriction site analysis of chloroplast DNA (Murakami and Schaal, 1994) and *rbcL* sequencing (Murakami, 1995). However, no molecular study for the whole family has previously been performed. Significant nucleotide sequence variation in the *rbcL* gene was found in the earlier study on *Hymenasplenium,* and even higher levels were to be expected for Aspleniaceae as a whole. It is widely believed that *rbcL* is a slowly evolving gene and not suitable for intrafamiliar or intrageneric phylogenetic analyses, but this is not in the case for the Aspleniaceae. Considerable amounts of sequence variations were observed even within a species complex, such as the *H. obliquissimum* (Hayata) Sugimoto et Kurata complex (Murakami et al., 1998) and the *A. nidus* L. complex (Murakami et al., 1999).

Materials and Methods

Plant Materials.—Fresh leaves of 19 species and 2 varieties of Aspleniaceae were collected, mostly in Japan. Only *A. ensiforme* and *A. nidus* were collected from Thailand and Laos, respectively. For *Hymenasplenium,* six representative Old and New World species that were shown to be distantly related in our earlier study (Murakami, 1995) were selected. In total, 25 species and 2 varieties (listed in Table 1) were used for molecular phylogenetic analyses of Aspleniaceae. Although the number of taxa sampled is too small to cover the entire spectrum of variation in Aspleniaceae, which has more than 700 species, it still covers most of the variation in leaf shape (simple to finely pinnatifid), presence or absence and various position of gemma, and rhizome shape (erect, ascending, to long creeping). The samples also contained representatives of all five of the segregate genera noted in the introduction. We do not necessarily recommend the adoption of all of these segregate genera; these names are used in this paper merely for convenience. Voucher specimens are deposited at the herbarium of the Faculty of Science, Kyoto University (KYO).

For allozyme analyses, *A. prolongatum* and all Aspleniaceae species that grow together with it on Yaku Island were collected (Table 2). For *A. ×kenzoi,* plant materials were obtained from cultivated stocks whose origins are known. They originated from at least two different localities: Yaku Island and Ohsumi Peninsula, both Kagoshima Prefecture, Japan. Ten individuals from each species, and five individuals of the hybrid were analyzed.

rbcL Sequencing.—Total DNAs were isolated from a single plant of each species using a modified CTAB method (Doyle and Doyle, 1987). When necessary, the DNAs were purified using a Qiagen column (tip 20) according to the vender's instructions. This purification procedure improved PCR amplification. Three fragments overlapping each other and covering most of the *rbcL* gene were amplified by PCR using two sets of primers developed by Hasebe et al. (1994) and one modified by ourselves. For amplifying the middle fragments of the *rbcL* gene (307–1016 nucleotide position of the tobacco *rbcL* gene), we designed a new set of primers, 5′-TATCCCTTAGACCTCTTCGAAGAAGGTTC

TABLE 1. Source of plant materials and voucher information for taxa sequenced for this investigation. All localities are in Japan, unless otherwise noted. Information for six species of *Hymenasplenium* sequenced for an earlier investigation (Murakami 1995) is also included. Collector abbreviations: SN, Satoru Nogami; KO, Koichi Oohora; RI, Ryuji Ito; YT, Y. Takahashi; NM, Noriaki Murakami; YH, Yuichi Higuchi.

Species	Locality	Voucher and DNA database accession number
Asplenium antiquum Makino (*Neottopteris antiqua* (Makino) Masam.)	Mt. Nishiyama, Hachijo Is.	*SN, RI & YT 34* (AB013235)
A. cardiophyllum (Hance) Baker (*Hymenasplenium cardiophyllum* (Hance) Nakaike; *Boniniella ikenoi* (Makino) Hayata)	Cult., Bot. Gard., Univ. of Tokyo	*NM 596921* (AB014706)
A. cheilosorum Kunze ex Mett. (*Hymenasplenium cheilosorum* (Mett.) Tagawa)	China, Xishuangbanna, Yunnan prov.	*NM and X. Cheng 93-C358* (AB014704)
A. ensiforme Wall. ex Hook. et Grev.	Thailand, Doi Inthanon	*Fukuoka et al. 93-T619* (AB014709)
A. griffithianum Hook.	Yakushima Is., Kagoshima Pref.	*NM J93-001* (AB014688)
A. hondoense N. Murak. et Hatanaka (*H. hondoense* (N. Murak. et Hatanaka) Nakaike)	Hayama, Kouchi Pref.	*NM 596920* (AB014705)
A. incisum Thumb.	Funada, Kiho, Mie Pref.	*SN & KO 9* (AB014697)
A. laetum Sw. (*H. laetum* (Sw.) N. Murak.)	Ecuador, Rio Palenque	*NM N293* (AB014707)
A. nidus L. (*N. nidus* (L.) J. Sm.)	Laos, Phatang, Vientiane	*Iwatsuki et al. 93-L585* (AB014687)
A. normale D. Don (var. *normale*)	Choshidani, Miyama, Mie Pref.	*SN, RI, YT & YH* 15 (AB014701)
A. normale var. *boreale* Ohwi ex Kurata	Kozuke, Shingu, Wakayama Pref.	*SN 22* (AB014702)
A. normale var. *shimurae* H. Ito	Choshidani, Miyama, Mie Pref.	*SN, RI, YT &YH 23* (AB014703)
A. obliquissimum (Hayata) Sugimoto et Kurata (*H. obliquissimum* Hayata)	Yakushima Is., Kagoshima Pref.	*NM 596902* (U30605)
A. oligophlebium Baker	Nabari, Mie Pref.	*SN 21* (AB014700)
A. prologatum Hook.	Funada, Kiho, Mie Pref.	*SN & KO 25* (AB014691)
A. pseudo-wilfordii Tagawa	Iwayadani, Kamikitayama, Nara Pref.	*SN 14* (AB014696)
A. riparium Liebm. (*H. riparium* (Liebm.) N. Murak.	Costa Rica, Virgen del Socorro	*NM & Grayum 281* (AB14708)
A. ritoense Hayata	Deai, Nchi-katuura, Wakayama Pref.	*SN & KO 27* (AB14692)
A. ruprechtii Kurata (*Camptosorus sibiricus* Rupr.)	Toyama Pref.	*NM 596918* (U30606)
A. sarelii Hook.	Nigishima, Kumano, Mie. Pref.	*SN & KO 11* (AB014693)
A. scolopendrium L. (*Phyllitis scolopendrium* (L.) Newm.)	Fukushima, Univ. of Tokyo	*Hasebe 26544* (U30607)
A. tenuicaule Hayata	Shingu, Wakayama Pref.	*SN & KO 10* (AB014694)
A. trichomanes L.	Nigishima, Kumano, Mie. Pref.	*SN & KO 13* (AB014698)
A. tripteropus Nakai	Tamakiguchi, Kumanogawa, Mie Pref.	*SN & KO 12* (AB014699)
A. wildfordii Mett. ex Kuhn	Funada, Kiho, Mie Pref.	*SN & KO 29* (AB014695)
A. wrightii Eat. ex. Hook.	Choshi-dani, Miyma, Mie Pref.	*SN, RI, YT & YH 3* (AB014690)
A. yoshinagae Makino	Choshi-dani, Miyma, Mie Pref.	*SN, RI, YT & YH 8* (AB014689)

TABLE 2. Voucher information of plant materials for allozyme analyses. All localities are in Japan. Collector abbreviations: NM, Noriaki Murakami; YH, Yuichi Higuchi.

Species	Locality	Voucher
Asplenium antiquum Makino (*Neottopteris antiqua* (Makino) Masam.)	Suzunoko, Yaku Is. Kagoshima Pref.	NM 93-J45
A. nidus L. (*Neottopteris* nidus (L.) J. Sm.)	Yaku Is. Kagoshima Pref.	NM 93-J46
A. griffithianum Hook.	Suzunoko, Yaku Is. Kagoshima Pref.	NM 93-J47
A. wrightii Eat. ex Hook.	Hana-agegawa, Yaku Is. Kagoshima Pref.	YH 1
A. prolongatum Hook.	Suzunoko, Yaku Is. Kagoshima Pref.	NM 26
A. cataractarum Rosenst. (*Hymenasplenium cataractarum* (Rosenst.) N. Murak.)	Suzunoko, Yaku Is. Kagoshima Pref.	NM 93-J48
A. wilfordii Mett. ex Kuhn	Hanaage-gawa, Yaku Is. Kagoshima Pref.	NM 93-J32
A. ×kenzoi Kurata	Cultivated, Bot. Gard., Univ. of Tokyo	NM 93-J50-J54

(TW-NP1, 307 Forward) and 5′-ACTGTTGTAGGTAAACTAGAAGGTGAACG (TW-2PR, 1016 Reverse), which eare more effective than those used by Hasebe et al. (1994), not only for Aspleniaceae but also for a wide range of vascular plants including angiosperms (Chayamarit 1997). The amplified fragments were isolated on agarose gels and purified using GeneCleanII (BIO 101 Inc.). Purified double-stranded DNA fragments were sequenced directly in both directions using an ALF autosequencer and AutoCycle sequence kit (Pharmacia) with FluoroPrime (the labeled primers for sequencing) having the same sequence that was used for amplification.

Assembly and alignments of the sequences were performed using the GENETYX computer program (Software Development, Tokyo). For phylogenetic analyses, PAUP version 3.1.1 (Swofford, 1993) was used. Most parsimonious trees were searched by a heuristic procedure with 100 random taxon-additions to find all equally optimal islands. This analysis was conducted with TBR, MULPARS, and unordered options in which all changes were equally weighted. In order to evaluate the internal support for monophyletic groups, bootstrap analyses (Felsenstein, 1985) were conducted with 1,000 replicates using heuristic searches.

Diplazium esculentum (Retz.) Sw. was used as an outgroup. We also tried to use *Athyrium otophorum* (Miq.) Koidz., *Dryopteris dickinsii* (Fr. et Sav.) C.Chr., and *Deparia petersenii* (Kunze) M.Kato as outgroups. Their validity as outgroups of Aspleniaceae was confirmed by Hasebe et al. (1994, 1995) when these authors conducted a molecular phylogenetic analysis of most fern families.

ENZYME ELECTROPHORESIS.—All procedures for enzyme electrophoretic analyses followed Shiraishi (1988). We analyzed seven enzyme systems: aspartate amino transferase (AAT), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), shikimate dehydrogenase (SKD), 6-phosphoglucose dehydrogenase (6PG), alcohol dehydrogenase (ADH), and phosphoglucomutase (PGM).

RESULTS

We determined 1,191 nucleotides of the *rbcL* sequences of 25 species and 2 varieties of the Aspleniaceae (Table 1). All sequences aligned easily without any insertions or deletions. The percentage difference of the nucleotides between the most distantly related species was about 7–9%. Percent sequence divergence among typical *Asplenium* species was 4–6%. Thus, *rbcL* sequence divergence was appropriate for phylogenetic inferences.

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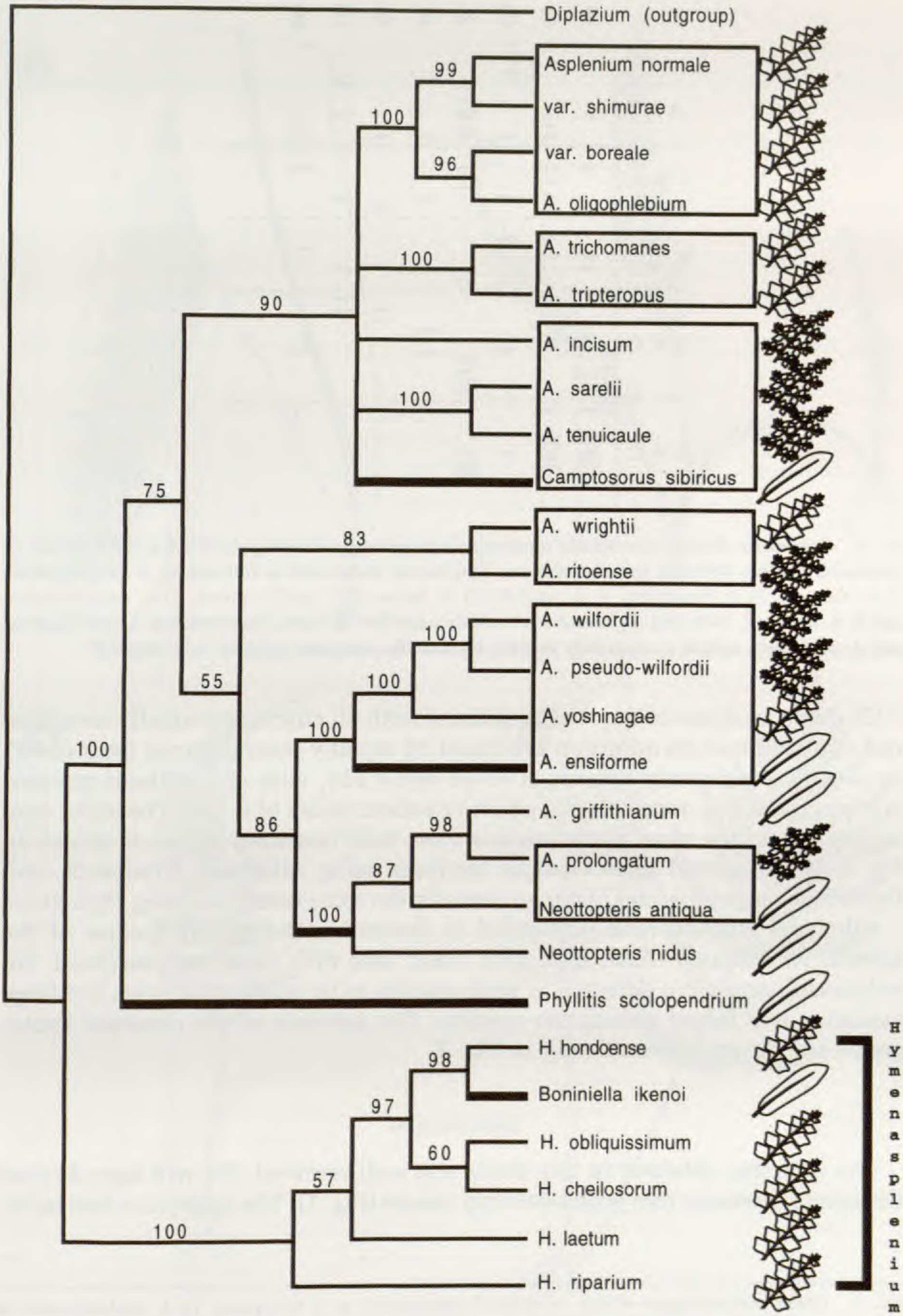
FIG. 1. Strict consensus tree of the most parsimonious trees of Aspleniaceae constructed based on *rbcL* sequences. The names of the segregate genera of Aspleniaceae are used only for convenience, and we do not recommend their use, except *Hymenasplenium* and *Phyllitis*. The leaf shapes (simple, once-pinnate, or finely pinnatifid) of each species or variety are shown on the right side of the tree. This molecular tree suggests that simple leaves evolved on at least 5 different lineages (shown by the thick lines). Groups of species with natural hybrids are boxed.


Diplazium (outgroup)
99
100
96
Asplenium normale
var. shimurae
var. boreale
A. oligophlebium
100
A. trichomanes
A. tripteropus
90
A. incisum
100
A. sarelii
A. tenuicaule
Camptosorus sibiricus
75
A. wrightii
83
A. ritoense
100
A. wilfordii
A. pseudo-wilfordii
55
100
A. yoshinagae
100
A. ensiforme
100
A. griffithianum
86
98
A. prolongatum
87
Neottopteris antiqua
100
Neottopteris nidus
Phyllitis scolopendrium
H. hondoense
98
Boniniella ikenoi
97
H. obliquissimum
60
H. cheilosorum
57
H. laetum
100
H. riparium
Hymenasplenium

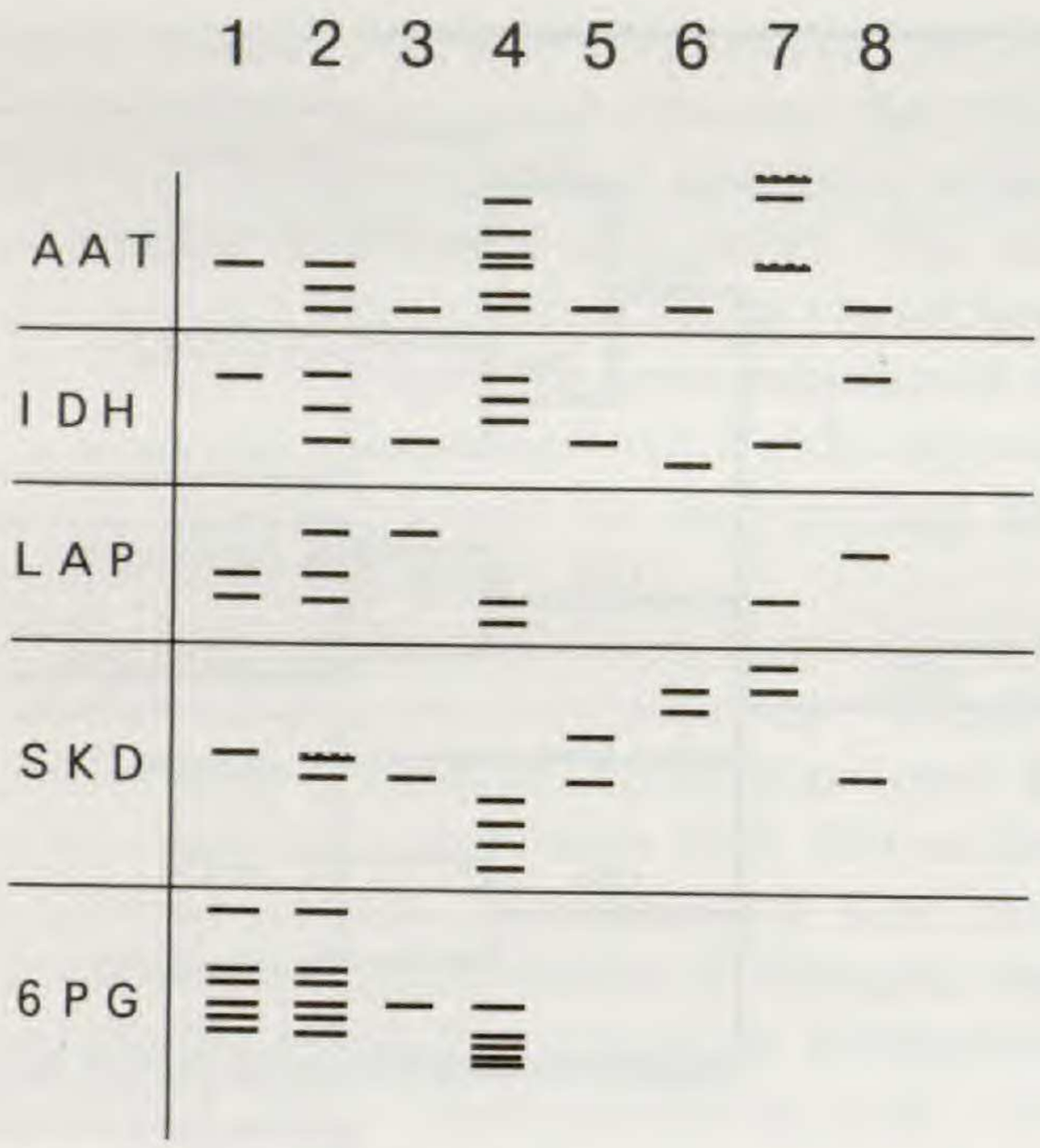


FIG. 2. Schematic view of zymograms showing electrophoretic banding profiles for eight species of *Asplenium* in five different enzyme systems. Species are numbered as follows: 1) *A. prolongatum*, 2) *A. ×kenzoi*, 3) *A. antiquum*, 4) *A. wrightii*, 5) *A. nidus*, 6) *A. griffithianum*, 7) *A. cataractarum*, and 8) *A. wilfordii*. Note that *A. ×kenzoi* has additive profiles of those characterizing *A. prolongatum* and *A. antiquum*, which is especially evident for dimeric enzymes, such as AAT and IDH.

Cladistic analyses of the *rbcL* sequences with all characters equally weighted and *Diplazium* as an outgroup produced 15 equally parsimonious trees of 585 steps with consistency indices of 0.638 and 0.525, with and without uninformative characters, respectively, and a retention index of 0.743. The strict consensus tree of the most parsimonious trees with bootstrap values is shown in Fig. 1. We also tried three cladistic analyses using *Athyrium, Dryopteris,* and *Deparia* as outgroups, and obtained virtually the same results as using *Diplazium.*

Allozyme analysis was performed to determine the parent species of the hybrid, *A. ×kenzoi*. AAT, IDH, LAP, SKD, and 6PG were well resolved. No polymorphisms were detected in each species as far as we examined, but large variation was found among the species. The patterns of the obtained zymograms are shown schematically in Fig. 2.

DISCUSSION

The *rbcL* tree obtained in this study was well resolved. We will here discuss the strict consensus tree with bootstrap values (Fig. 1). The molecular tree most-

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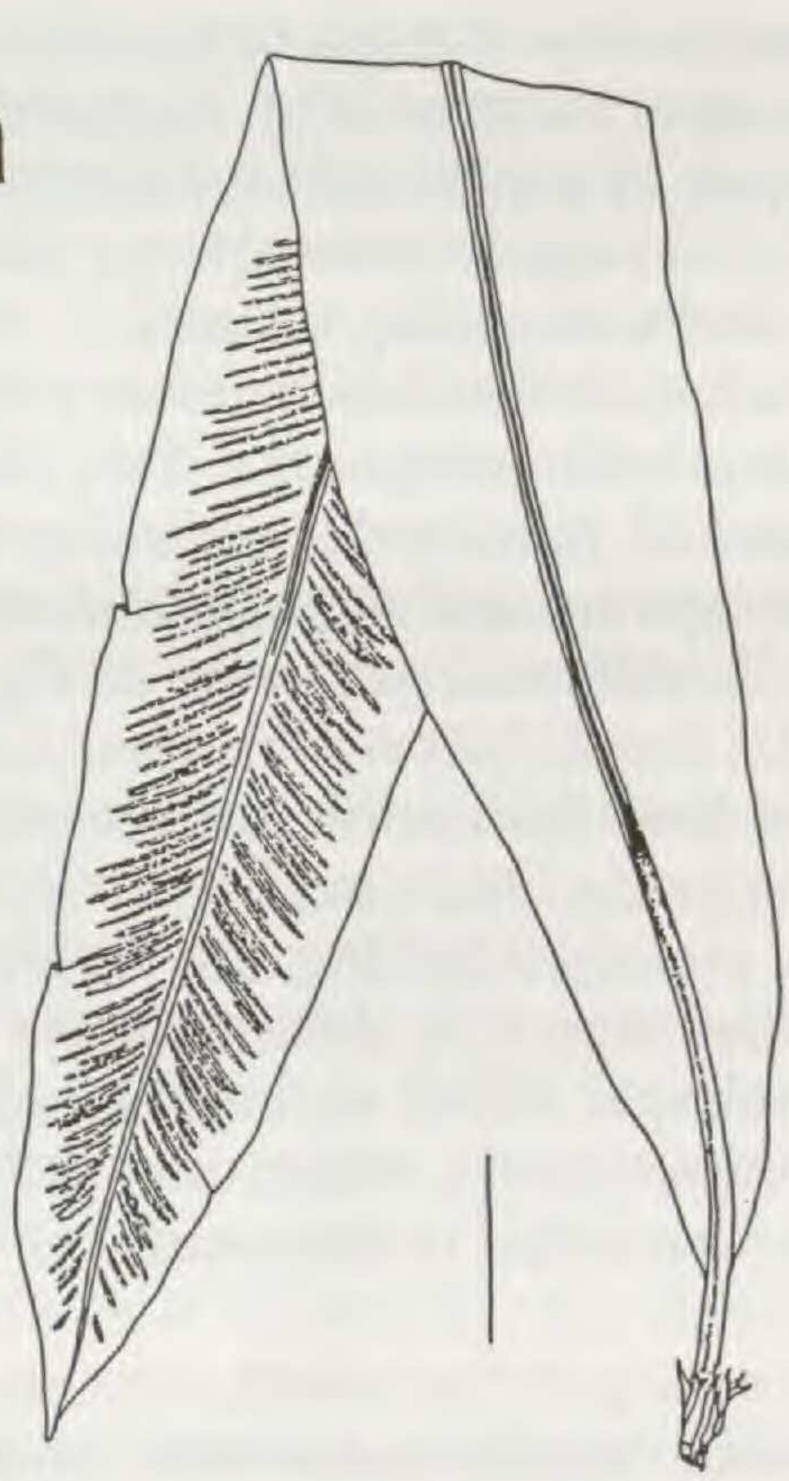
FIG. 3. Gross morphologies of four species of *Asplenium*. a) *A. antiquum*. b) *A. prolongatum*. c) *A. ×kenzoi*. d) *A. wrightii*. Scale Bar = 5 cm. Note that *A. ×kenzoi* (c) was believed by earlier workers to represent a hybrid of *A. prolongatum* and *A. wrightii* (b × d), but in this study it was shown to be a hybrid between *A. prolongatum* and *A. antiquum* (b × a).


a


b


c


d

ly correlates well with overall morphological similarities. Groups of species that were very similar morphologically, such as the three varieties of *A. normale, A. oligophlebium, A. trichomanes,* and *A. tripteropus, A. sarelii* and *A. tenuicaule,* and *A. wilfordii, A. pseudo-wilfordii,* and *A. yoshinagae,* were always found together in clades of high statistic confidence (100% bootstrap values).

However, we also found several anomalous affinities between species, which were not expected by gross morphology and thus were noteworthy. Two *Neottopteris* species, *N. antiqua* (=*A. antiquum*) and *N. nidus* (=*A. nidus*), or the "birds nest ferns," formed a clade with *A. prolongatum* and *A. griffithianum* in our *rbcL* tree. Their gross morphologies are quite different, as shown in Fig. 3. The segregate genus *Neottopteris* is defined by its simple leaves with specialized anastomosing venation in which parallel veins from the midrib are connected only at the leaf margin. *Asplenium griffithianum* also has simple leaves, but with venation that is completely free. As for *A. prolongatum* (Fig. 3b), its leaves are pinnatifid 3–4 times and have gemma at their tips. It is different from the other three species of the clade, which have simple leaves without gemmae. From a phylogenetic point of view, the genus *Neottopteris,* which was defined only by its special simple leaf, is paraphyletic according to the results of this study.

A similar situation was found for two other segregated genera, *Camptosorus* and *Boniniella.* Both are partly defined by their simple leaves with anastomosing veins. However, they are found in two different clades that contain pinnatifid species with free veins. There is therefore no merit in recognizing these genera.

According to our molecular tree and the most parsimonious reconstruction of leaf shape evolution, simple leaves may have evolved at least five times from pinnatifid leaves in Aspleniaceae (in the two different evolutionary lineages leading to *Phyllitis scolopendrium, Camptosorus sibiricus,* and *A. ensiforme,* in addition to *Neottopteris* and *A. griffithianum,* and also *Boniniella* (see Fig. 1). Thus, simple leaves or even pinnatifid ones do not reflect phylogeny in the Aspleniaceae.

Hymenasplenium is defined by having creeping rhizomes with dorsiventrality (Hayata, 1927). *Boniniella* also has rhizomes of the same construction as those of *Hymenasplenium* (Hayata, 1927) and they share the peculiar chromosome base number x = 39, in contrast to x = 36, which is found in almost all other members of Aspleniaceae (Mitui et al., 1989, Kato et al., 1990). *Hymenasplenium laetum* and *H. riparium* from the New World tropics, the three Asian species of *Hymenasplenium,* and *Boniniella* (=*Hymenasplenium cardiophyllum*) were found to be most distantly related within *Hymenasplenium* by Murakami (1995). Our present molecular tree clearly supports the hypothesis that species with dorsiventral creeping rhizomes, including both Old and New World species of *Hymenasplenium,* together with *Boniniella,* are a monophyletic group. Moreover, this group is shown to be sister to the other members of Aspleniaceae in our *rbcL* tree (Fig, 1). Most pteridologists have considered the *Hymenasplenium* group to be of recent origin, perhaps from the *A. normale* and *A. trichomanes* group (Holttum, 1954; Iwatsuki, 1975). However, it is one

the oldest groups within the family and has no affinities to any of the other members of *Asplenium*. It is also easily definable phenetically by its dorsiventral creeping rhizomes and the peculiar chromosome basic number x = 39. Thus, we recommend use of the genus name *Hymenasplenium* for this group even before the other members of Aspleniaceae (especially *Asplenium*) are appropriately subdivided into several monophyletic genera.

The monotypic genus *Phyllitis* was defined by having a special sori construction: the two closest sori face each other and overlap. This character is not clear enough to define a genus, and *Phyllitis* is not widely accepted. However, our molecular tree shows that *Phyllitis* is sister to *Asplenium* (including *Camptosorus* and *Neottopteris*) and is the most distantly related to all other Aspleniaceae species when the *Hymenasplenium* group is excluded. Thus, *Phyllitis* should also be accepted as a genus in Aspleniaceae, but it should be carefully redefined morphologically.

Natural hybrids of Aspleniaceae became famous after the excellent work of Wagner (1954) on North American species, which first demonstrated reticulate evolution in plants. Many putative nonfertile natural hybrids in *Asplenium* have been reported from Japan, such as *A. ×kitazawae* Kurata & Hutoh (*C. sibiricus × A. sarelii*), *A. ×kobayashii* Tagawa (*C. sibiricus × A. incisum*), *A. ×kenzoi*(*A. prolongatum × A. wrightii*), *A. ×shikokianum* Makino (*A. wrightii × A. ritoense*), *A. ×iidanum* (Kurata) Shimura & Takiguchi (*A. pseudo-wilfordii × A. yoshinagae*), *A. tenuicaule × A. sarelii, A. trichomanes × tripteropus,* and *A. normale* var. *normale* × var. *boreale* (Iwatsuki, 1995). When we locate the parents of these natural hybrids on the *rbcL* tree, they are typically very closely related. For example, although the morphologies of *A. wrightii* and *A. ritoense* are quite different, their hybrid, *A. ×shikokianum,* has been found in numerous localities where the two parental species grow together, all over Japan (Iwatsuki, 1995) as well as in China (Mt. Emei). *Asplenium wrightii* and *A. ritoense* are sister species in our molecular tree.

Similarly, *Camptosorus sibiricus* has unusual simple leaves that are different from those of all other Aspleniaceae species (which was used by earlier botanists as justification for its status as a segregate genus), but it often hybridizes with *A. incisum, A. sarelii,* and other pinnatifid leaved species of *Asplenium.* This "walking fern" was shown to be closely related to the species with which it often hybridizes.

One apparent exception is *A. ×kenzoi* (Fig. 3c), which was described from Yaku Island by Kurata (1962) as a hybrid between *A. prolongatum* and *A. wrightii* (Fig. 3d), which are relatively distantly related in *Asplenium* according to our molecular tree. *Asplenium ×kenzoi* is evidently a hybrid because of its sterile spores and distorted leaf shape. It is also certain that one of its parents is *A. prolongatum* because of strong morphological similarities (Fig. 3). We determined about 500 *rbcL* nucleotide sequences of 5 cultivated individuals of *A. ×kenzoi* that originated from at least two different localities, Yaku Island and the Ohsumi Peninsula, in Kagoshima Prefecture. Their sequences are exactly the same as those of *A. prolongatum* and different from those of all other Aspleniaceae species so far determined. This result indicates

that the chloroplast (presumably maternal) parent of *A. ×kenzoi* is *A. prolongatum.* The maternal inheritance of chloroplast DNA, which contains the *rbcL* gene, was reported for *Asplenium* (Vogel et al., 1998) as well as for some other ferns (Gastony and Yatskievych, 1992). We had suspected previously that *A. wrightii,* which had been considered the other putative parent by Kurata (1962), was perhaps not involved. Thus, we collected all possible parental species among Aspleniaceae that coexist with A. *prolongatum* on Yaku Island to find the paternal parent of *A. ×kenzoi.* (Table 2). The zymograms indicated that the second parent of *A. ×kenzoi* is in fact *A. antiquum,* not *A. wrightii,* which had no strong relationship to the hybrid. Especially for dimeric enzymes like AAT and IDH, the hybrid bands of *A. prolongatum* and *A. antiquum* were seen additively in *A. ×kenzoi.* These results support the hypothesis that in Aspleniaceae only closely related species hybridize naturally. Moreover, two species that hybridize often may be closely related even if they are very different in morphology. Our *rbcL* tree of selected species of Aspleniaceae suggested an evolutionary origin of a hybrid that might have never been expected from morphological comparisons.

In this study, we examined only 3% of all Aspleniaceae species, and although the results are still preliminary, they provide many interesting implications for understanding the phylogeny of the family. We will continue this type of molecular study of the Aspleniaceae and expand our data set in the future by collecting plant materials from all over the world to represent the diversity of the family.

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